DEVELOPING A HYBRID BREEDING SYSTEM FOR TURNIP RAPE

DOCTORAL THESIS

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ACADEMIC DISSERTATION

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The following table presents the contributions of the authors to the original articles of this thesis:

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ABBREVIATIONS

BC	backcross
BAC	bacterial artificial chromosome
CMS	cytoplasmic male sterility
FISH	fluorescence in situ hybridization
GISH	genomic in situ hybridization
NE	normalized gene expression value
PCR	polymerase chain reaction
PPR	pentatricopeptide-repeat
qPCR	quantitative polymerase chain reaction
Rf	fertility restorer
ROC	receiver operating curve

Keywords: Turnip rape, *Brassica rapa*, hybrid breeding, synthetic, composite hybrid, commercial heterosis, CMS/Rf hybrid system, fertility restoration, radish, *Raphanus sativus*, Kosena Rfk1 gene, Ogura CMS, TaqMan qPCR, GISH, BAC-FISH

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ABSTRACT

Spring turnip rape (*Brassica rapa* ssp. *oleifera*) is the major oilseed crop cultivated for the production of vegetable oil and animal feed protein in Finland. The earliness of turnip rape favours its cultivation in northern climates, but its seed yield is low. Hybrid breeding has been utilized in many agricultural crops to improve the seed yield. The exploitation depends on the degree of heterosis that a specific crop shows and on the requirement of a functional hybrid system to cross the parent lines in the production of F1 hybrid seed. In this thesis, the possibility to advance hybrid breeding in developing higher-yielding spring turnip rape cultivars was studied.

The effects of heterosis on spring turnip rape were tested using synthetics and composite hybrids. All the tested material had low erucic acid and glucosinolate content and was well adapted for cultivation in northern climates. The Ogu-INRA CMS/Rf hybrid system, originally transferred from Japanese radish (*Raphanus sativus*), was selected for the study as one of the most promising F1 hybrid systems for turnip rape. The Ogura CMS produce stable, male-sterile lines in turnip rape. To generate a functional, fertility-restoring male line, the Kosena fertility-restoring gene *Rfk1*, originating from radish and homolog of the Ogura *Rfo* gene, was transferred from oilseed rape (*Brassica napus*) into turnip rape through interspecific crosses followed by traditional backcrossing. The TaqMan-based qPCR method was used to distinguish homozygous (Rfk1,Rfk1) turnip rape restorer plants from heterozygous plants. The physical location of the radish introgression carrying the *Rfk1* gene in turnip rape genome was investigated using GISH and BAC-FISH.

Spring turnip rape showed heterosis in increased seed yield, accompanied by increases in oil and protein yield but not in oil or protein content. Composite hybrids exhibited significantly higher seed yields than open-pollinated cultivars. Both composite hybrids and synthetics had better resistance to lodging than commercial cultivars. It was clearly important to test parental combinations. Considerable variation in commercial heterosis for seed yield was observed between the tested combinations within the groups both in synthetics and composite hybrids. The maximum yield increase achieved was 18% in synthetics and 23% in composite hybrids. The Kosena fertility-restoring gene *Rfk1* was successfully transferred from oilseed rape into turnip rape. The TaqMan qPCR method proved useful in selecting homozygous restorer plants before flowering. Interpollination of selected plants instead of inbreeding is a benefit in cross-pollinating crops that suffer from inbreeding depression. The *Rfk1* gene was able to restore the fertility of turnip rape with Ogura CMS, but the trait was unstable in the turnip rape genome. The *Rfk1* gene was localized on an additional radish chromosome in the A genome of turnip rape. The BAC64 clone, carrying the *Rfo* locus, homolog to the *Rfk1*, identified the locus both in the additional radish

chromosome and in the turnip rape chromosome (A09). The high homology of this locus between radish and turnip rape and its location in subterminal part of the chromosome in both genomes would facilitate the transfer of the fertility-restoring trait from radish to turnip rape. It was concluded that additional breeding techniques such as changing the ploidy level or using irradiation to increase the recombination activity between nonhomologous R and A genomes may be required.

Even though 100% F1 hybrids were not tested here, the clear evidence of heterosis in seed yield supports the use of hybrid breeding in producing higher yielding turnip rape cultivars. The breeding work towards a stable, fertility-restoring male line for the Ogu-INRA CMS/Rf hybrid system is challenging but feasible, owing to the homology of the fertility-restoring region found between radish and turnip rape genome and its location in the distal part of the chromosomes in both genomes. To have a functional Ogu-INRA CMS/Rf hybrid system for turnip rape, it seems ideal if the homologous chromosomal area in the A genome could be substituted with the radish chromosome area having the restorer gene.

1 INTRODUCTION

1.1 TURNIP RAPE AS AN OILSEED CROP IN FINLAND

Turnip rape (*Brassica rapa* L. ssp. *oleifera* (DC.) Metzg.) belongs to the large genus *Brassica,* being a diploid species carrying the A genome (2n=20, AA). The *Brassica* genus has the greatest economic importance within the *Brassicaceae* family since it includes many important oilseed and vegetable crop species. The genomic relationship of the *Brassica* species was described in the classical triangle of U in 1935 (referred by Olsson and Ellerström 1980), which shows that there are three basic diploid species *B. rapa* (2n=20, AA), *B. oleracea* (2n=18, CC) and *B. nigra* (2n=16, BB) and three allotetraploid species *B. napus* (2n=38, AACC), *B. juncea* (2n=36, AABB) and *B. carinata* (2n=34, BBCC). The allotetraploid species most likely originated from diploid ancestors through natural hybridization followed by spontaneous chromosome doubling.

The main three species cultivated worldwide as oilseed crops are *B. napus*, *B. rapa*, including turnip rape and Indian sarson ecotypes, and *B. juncea*. Rapeseed production, including all these mentioned species, is worldwide the fourth most produced oilseed crop after soybean, oil palm and coconuts and in the European Union, rapeseed is the most important oilcrop, harvested from 8.8 M ha in 2010 (http://faostat.fao.org). Oilseed rape (*Brassica napus* L.) is the most important and most widely grown *Brassica,* being superior in both yield and quality, whereas turnip rape is important in some northern latitudes. It is the most cold-hardy of the *Brassica* oilseed species and has a relatively high growth rate under low temperatures (Buzza 1995). Turnip rape has earlier maturity than the earliest oilseed rape cultivars and shows better yield stability in cool growing conditions. Winter cultivars exist of both oilseed rape and turnip rape, but their rather low winter hardiness limits their cultivation in Finnish climate.

Spring turnip rape is the main oilseed crop cultivated in Finland for the production of vegetable oil and animal feed protein. It became a part of our cultivation practices during the 1980s, when the growing area stabilized to 60-80 thousand hectares. This represents around 3% of the country's cultivated area. During the 21st century, the cultivation area has been approximately 83 000 hectares, and the yearly seed production around 110 million kg (http://www.maataloustilastot.fi/en/utilised-agricultural-area). Turnip rape and oilseed rape oil are considered among the healthiest vegetable oils because the fatty acid composition is closest to the optimum to meet the basic requirements for essential fatty acids in the human body (Seppänen-Laakso et al. 2010). Rapeseed meal has a good balance of essential amino acids, so it is valued as high nutritional quality protein for animal feed (Bell 1995). The oil and protein content of turnip rape varies in official yield trials between 39.5 and 42.9% and between 22.5 and 23.5% respectively (Kangas et al. 2010).

The average yield of turnip rape over the last ten years has been modest, only 1330 kg ha^{-1}, and is slightly decreasing (http://www.maataloustilastot.fi/en/crop-production-statistics). The work towards higher seed yields and yield stability has been stated as one of the main goals in oilseed crop production in the national grain production strategy for 2006-2015 (Ministry of Agriculture and Forestry 2006).

1.2 HYBRID BREEDING

Hybrid breeding has been utilized in developing more productive cultivars since Shull published his observations on crossing and inbreeding maize plants at the beginning of the 19th century (Sleper and Poehlman 2006). Hybrid maize is one of the greatest success stories of plant breeding. The introduction of hybrid breeding has been estimated to increase maize production up to 75% (Chrispeels and Sadava 2003). Hybrid breeding is based on the phenotypic superiority of heterozygote F1 generation plants. The F1 hybrid refers to the first generation of a cross between two different parent lines that are almost homozygous. When crossing two nearly homozygous parent lines the offspring is very homogeneous but highly heterozygous. When the parent lines have good combining ability, it leads to vigorous but at the same time very uniform growth, benefiting the hybrid cultivars. The commercial utilization of hybrid breeding in agricultural crops depends on the degree of heterosis or hybrid vigour that a specific crop shows which differs between crop species (Duvick 1999). Another requirement is a functional and economically viable seed production system; the way to cross parent lines to produce F1 hybrid seed in commercial scale. Agricultural crops that are commonly grown as hybrids include Brussels sprouts (*Brassica oleracea* Gemmifera group), kale (*Brassica oleracea* Acephala group), maize (*Zea mays* L.), onion (*Allium cepa* L.), rapeseed (*Brassica napus* L.), sorghum (*Sorghum bicolor* L.), sunflower (*Helianthus annuus* L.), tomato (*Solanum lycopersicum* L.) (Brown and Caligari 2008) and rice (*Oryza sativa* L.) (Virmani and Kumar 2004).

1.2.1 Heterosis

Heterosis, or hybrid vigour, describes the superior performance of heterozygous F1 hybrid plants over their homozygous parental lines. Heterosis usually results from a cross between two genetically different, highly inbred lines. The level of heterosis often increases when the genetic distance of the parent lines increases (Chen 2010). Self-pollination of hybrid plants leads to the gradual decreasing of heterosis over the number of selfing generations. Especially in cross-pollinating crops, this lead to inbreeding

depression, which is the opposite to heterosis. Usually heterosis is greater in cross-pollinating species than in self-pollinating species (Chen 2010) as a result of the important role of the heterozygosis in fitness of natural populations to variable environmental conditions (Shi et al. 2011). Also in interspecific crosses or allopolyploids, that contain two or more sets of genetically distinct chromosomes, the level of heterosis is high (Chen 2010).

Although heterosis has been utilized commercially in cultivar breeding in many plant species over the decades, the genetic and molecular basis of heterosis is still not understood (Hochholdinger and Hoecker 2007; Lippman and Zamir 2007; Chen 2010). There are three main hypotheses for heterosis, but none of them explains the phenomenon completely (Banga 1998; Crow 1999; Filho 1999; Goodnight 1999). The dominance hypothesis is based on superiority of the dominant alleles over the recessive ones. The theory is based on the assumption that dominant alleles contribute vigour and growth, whereas recessive alleles may be neutral, harmful, or deleterious to the individual (Sleper and Poehlman 2006). The second model is the over-dominance hypothesis, which claims that contrasting alleles in single locus produce different favourable effect on the plant. It is based on interaction of alleles at the same locus and the phenomenon of heterozygote is superior over the homozygote (Sleper and Poehlman 2006). The third model is the epistasis hypothesis, where heterosis is a result from epistatic interactions between different loci (Goodnight 1999). The allelic substitution at one locus affects of allelic substitution at all other loci and the interaction between non-allelic genes causes the superior phenotypic expression in hybrids (Hochholdinger and Hoecker 2007; Fievet et al. 2010).

Heterosis involves multiple quantitative traits expressed together, so the studies on QTLs (quantitative trait locus) associated with heterosis have been numerous (Hochholdinger and Hoecker 2007; Lipman and Zamir 2007; Radoev et al. 2008; Basunanda et al. 2010; Fievet et al. 2010; Shi et al. 2011). Identification of the genes and QTLs behind heterosis is challenging because of the complexity of the interaction between phenotypic components, which are also affected by the environment (Hochholdinger and Hoecker 2007; Lipman and Zamir 2007). Heterosis varies between years and locations, so it should be tested in field conditions (Shi et al. 2011).

1.2.2 Combining abilities

In hybrid breeding, inbred parental lines that combine well to express heterosis are needed. In cross-pollinating crops, the homozygous parental lines are made by inbreeding segregating populations for several generations. The combining ability of the parent lines is evaluated by screening their performance in hybrids. Combining ability is classified into two categories; the general combining ability (GCA) represents the average performance of the parent line in hybrid combinations, and the specific combining ability (SCA) is

the contribution of the parent line to hybrid performance in a cross with a specified inbred line (Sleper and Poehlman 2006). Both GCA and SCA of the parent lines are used when selecting inbred lines for the hybrid breeding program. In breeding programs, it has been observed that some parent lines combine well with a large number of other parent lines and some parent lines combine well only with few or none of the other inbred lines. Usually few breeding lines having a good GCA are chosen as a test parents in a breeding programs. The aim is to evaluate the new breeding material in early generations to focus breeding work on the most promising parent lines.

1.2.3 Expression of heterosis

The level of heterosis is usually presented as a mid-parent value, a high-parent value or a commercial heterosis. Mid-parent value refers to the productivity of F1 hybrid over the average of its parents, whereas in the high-parent value the productivity is calculated over the better parent. For the commercial heterosis, comparisons are made with the competing commercial cultivar. Hybrid vigour means generally increase in plant size or rate of growth (Duvick 1999), which is observed in crops as increased biomass production, faster development, higher leaf area index, increase in root growth and in seed yield (Banga 1998). In most cases, heterosis improves seed yield and the worldwide yield benefit due to hybrid varieties is estimated to be 10% in maize, 19% in sorghum and 30% in sunflower (Duvick 1999).

Heterosis for seed yield has been reported in all main oilseed *Brassica* species, the largely self-pollinating allopolyploids *B. napus* and *B. juncea* and cross-pollinating diploid *B. rapa* (Fu and Yang 1998). Oilseed rape is the most widely grown and intensively studied of the *Brassica* species, and significant heterosis for seed yield was already reported during the 1980s (Sernyk and Stefansson 1983; Grant and Beversdorf 1985; Brandle and McVetty 1989). According to these studies, the average high parent heterosis for seed yield was 30% for spring and 50% for winter oilseed rape. Basunanda et al. (2010) reported an average yield increase of 11% in winter oilseed rape hybrids compared to open-pollinated cultivars in practical experience in Germany. In addition, heterosis has been reported for seedling biomass, growth, plant height, lodging resistance and yield components, such as 1000 seed weight, seeds per silique and number of siliques per unit area (Sernyk and Stefansson 1983; Brandle and McVetty 1990; Radoev et al. 2008; Basunanda et al. 2010). Heterosis for oil or protein content, however, is seldom observed (Brandle and McVetty 1990).

In turnip rape, the few studies on heterosis that have been conducted have shown significant heterosis for seed yield (Hutcheson et al. 1981; Schuler et al. 1992; Falk et al. 1994, 1998). Hutcheson et al. (1981) found 46% commercial heterosis for seed yield in hybrid *B. rapa* ssp. *sarson* (R-500) x *B. rapa* ssp. *oleifera* over the cultivar ‘Candle’. However, in those

experiments the pollen source of *B. rapa* ssp. *oleifera* parent was not controlled and the erucic acid level was the only indicator used for hybrid seed. These weaknesses in the experimental setup decrease the reliability of the results. Schuler et al. (1992) crossed *B. rapa* ssp. *oleifera* Canadian cultivar 'Tobin' with 19 Canadian and European strains and reported an average of 18% mid-parent heterosis for seed yield, 17% heterosis for oil yield and 7% heterosis for plant height. No or slightly negative heterosis was found for oil content, days to flower and maturity and single seed weight. Falk et al. (1994) tested heterosis in *B. rapa* hybrids using four cultivars adapted to western Canada. Reciprocal crosses were made to test the possible maternal and paternal heterosis. During three years of testing, an average of 13% (1.7 – 27.1%) mid-parent heterosis was observed for seed yield, and reciprocal differences were reported. One cultivar indicated a positive maternal effect on hybrid yields. Oil content and days to maturity did not show heterosis.

1.2.4 Utilizing heterosis in variety breeding before F1 hybrids

There is currently no functional hybrid seed production system available for turnip rape. In both oilseed rape and turnip rape, synthetics and composite hybrids have been developed and used on the commercial scale. Often they are used as a temporary solution to increase heterosis until a functional hybrid system has been developed.

Synthetic varieties in turnip rape are developed by mechanically mixing two or three relatively homozygous and uniform parental lines (Falk 1991; Buzza 1995). This so-called Syn0 generation is planted in isolation for random intercrossing of the parents to produce Syn1 generation seed. The level of heterosis is highest in Syn1 (Falk and Woods 2003) so it is the generation most often cultivated. During the succeeding generations of open pollination, called Syn2, Syn3, etc., the level of heterosis gradually decreases although all the synthetic generations yielded significantly more than the parents (Falk and Woods 2003). In the case of two-parent synthetics, when the parent lines have equal growth vigour, good outcrossing abilities and the same flowering time, the proportion of F1 hybrids can be as high as 50% and the proportion of each inter-parent sibs around 25% (Falk et al. 1998). Turnip rape has an advantage in producing synthetics in comparison with oilseed rape because its strong self-incompatibility system ensures a high percentage of cross-pollination between the parent lines as well as predictable and repeatable production of the Syn1 generation. The concept of using synthetics in turnip rape cultivation was represented by Falk (1991). Synthetics were found as preferred varietal type due to heterosis for seed yield and cost effective seed production. When comparing the heterosis in *B. rapa* hybrids and synthetics over the parents, Falk et al. (1998) observed 25% and 23% mid-parent heterosis for seed yield respectively.

Composite hybrids are generated by mixing seeds of male-sterile F1 hybrid plants (75-80%) with the seeds of fertile plants (20-25%) as a pollen source. This increases the proportion of F1 hybrids by 25% comparing to synthetics. Composite hybrids have been in commercial use both in oilseed rape and turnip rape. The first winter oilseed rape composite hybrid, Synergy (INRA), was registered in France in 1994 and the first turnip rape composite hybrid, Pouta (Mildola Ltd.), was registered in Finland in 2001 (Plant Variety Board 2001). Yield increases of 22% for Synergy (Renard et al. 1995) and 8-10% for Pouta (Kangas et al. 2002) were reported over the competitive pure line or open-pollinated cultivars. In oilseed rape, the performance of composite hybrids varied according to the weather conditions during flowering period. Kightley (1999) demonstrated that composites were more sensitive to cold and wet conditions during pollination, resulting in decreased seed set. The composite hybrid Pouta was withdrawn from the markets already in 2002 due to company rearrangements, which ended the further development of composite hybrids for Finnish markets.

1.2.5 Hybrid seed production systems

Some form of male-sterility is necessary to ensure cross pollination in F1 hybrid production. In genus *Brassica*, several hybrid seed production systems have been developed, but few are utilized commercially. The most commonly used hybrid systems are based on cytoplasmic male sterility (CMS) and genic or nuclear male-sterility (GMS/NMS). In oilseed rape, one of the nuclear male-sterility systems successfully used in European countries is based on a spontaneous mutant, Male Sterility Lembke (MSL) (Friedt and Snowdon 2009). In turnip rape, nuclear male sterility also exists, but as it is a recessive gene, it is necessary in hybrid seed production to rogue the 50% of male fertile plants from among the male-sterile plants, which is not economically or technically feasible (Velasco and Fernandez-Martinez 2009). Turnip rape, unlike oilseed rape, is self-incompatible, which could be used in hybrid seed production, but then introduces the problem, of how to economically maintain the parent lines.

1.2.5.1 CMS/Rf systems

Cytoplasmic male sterility (CMS) is based on incompatibility between the nuclear and mitochondrial genomes, which leads to the inability to produce functional pollen (Hanson and Bentolila 2004; Touzed and Budar 2004). CMS is rather common, having been found in over 150 plant species (Schnalbe and Wise 1998; Wise and Pring 2002). It has also been developed by using wide hybridization combining nucleus and cytoplasm from different species

(called alloplasmy) through backcrossing or protoplast fusion (Kaul 1998). The CMS-associated genes are often chimeric mitochondrial genes and thus, maternally inherited (Hanson and Bentolilla 2004; Touzet and Budar 2004). In the CMS system, utilized in hybrid seed production, the male-sterile line is called the A-line or female line. To maintain the male-sterile A-line, it is crossed in seed production with the B-line or maintainer line having the same genotype as the A-line, but normal cytoplasm without the sterility-inducing mitochondrial genes. To produce fully fertile F1 plants, the A-line is crossed with the fertility-restorer (Rf) line, also called the R-line or male line. The fertility restorer carries nuclear genes that prevent the action of the male-sterility inducing mitochondrial genes. Most of the known restorers affect the transcript profile or the protein accumulation of the CMS-associated locus (Hanson and Bentolilla 2004). Several Rf genes that have been cloned encode mitochondria-targeted pentatricopeptide-repeat (PPR) proteins, including those from petunia (*Petunia hybrid* L.), rice (*Oryza sativa* L.), radish (*Raphanus sativus* L.) and sorghum (*Sorghum bicolor* L.) (for review, see Chase 2006). Rf genes encoding PPR proteins are carried on complex loci that contain several closely related genes, most of which are unable to restore fertility except the one or ones that are responsible for restoration (Mora et al. 2010).

In *B. rapa*, a number of CMS systems have been identified. The Polima CMS (*pol* CMS) system, found in spontaneous male-sterile plants of a *B. napus* cultivar in China (Fu 1981), is utilized in commercial hybrid seed production of oilseed rape. *Pol* CMS and its nuclear restorer gene *Rfp* have been successfully transferred from *B. napus* to *B. rapa* (Verma et al. 2000; Formanova et al. 2006). According to Verma et al. (2000), the stable maintainers and restorer lines were identified, but commercialization of the *pol* CMS/Rf system in *B. rapa* hybrid seed production has not been reported. The problem in using *pol* CMS/Rf is its sensitivity to temperature. In different genotypes and at different temperatures, partial male fertility has appeared (Yang et al. 2006; Fan et al. 2007). The petals of the *pol* CMS plants are small and flowers open widely, so it is possible for bees to visit the flower nectaries from the side without pollinating the stigma. Incomplete pollination lowers the seed yields. Most of the reported *B. rapa* CMS lines have been produced through interspecific or intergeneric crosses, where the nucleus of *B. rapa* was combined with cytoplasm from other species such as *Diplotaxis muralis* (*mur* CMS) (referred by Fu and Yang 1998), *B. tournefortii* (*tour* CMS) (referred by Fu and Yang 1998), *B. oxyrrhina* (*oxy* CMS) (Prakash and Chopra 1990), *Eruca sativa* (Matsuzawa et al. 1999) or *Enarthrocarpus lyratus* (*lyr* CMS) (Deol et al. 2003). The utilization of these CMS systems in *B. rapa* hybrid seed production has, however, been limited by the lack of stable maintainer or restorer lines and other negative effects. Lack of maintainer genes has been reported in *mur* CMS and *lyr* CMS (Fu and Yang 1998; Deol et al. 2003). The *B. rapa* CMS carrying *Eruca sativa* cytoplasm has some negative effects caused by intergeneric hybridization that require further selection for higher female fertility and complete nectary development

(Matsuzawa et al. 1999). *Oxy* CMS is associated with reduced height and chlorosis (Prakash and Copra 1990). The limitations of the *tur* CMS system are the incomplete restoration of fertility, malformed flowers, and reduced number of nectaries (referred by Banga 1993). The *hau* CMS, found in a mutant of *B. juncea*, has been transferred to a few *B. rapa* vegetables, and the absence of a restorer line limits its usefulness in hybrid breeding (Wan et al. 2008).

1.2.5.2 Ogura/Kosena CMS/Rf

The Ogura CMS/Rf system is the most studied of the CMS systems as it provides highly stable male sterility in different environments, and an effective restorer gene was found allowing hybrid seed production. The Ogura CMS was found in Japanese radish (*Raphanus sativus* L.) by Ogura (1968) and it was first transferred to *B. napus* and *B. oleracea* through intergeneric crossing followed by successive backcrossing (Bannerot et al. 1974, 1977). The Ogura CMS has stable male-sterile lines, but these suffered from chlorophyll deficiency and reduced number of nectaries. These problems were later reduced through protoplast fusion (Pelletier et al. 1987). Ogura CMS is controlled by a mitochondrial gene, *orf138*, which induces abnormal flower development and prevents the production of functional pollen (Bonhomme et al. 1992; Krishnasamy and Macaroff 1994). In order to use the Ogura CMS in hybrid production, the fertility-restoring genes were found and introgressed from radish to *B. napus* by intergeneric crosses between an Ogura CMS *B. napus* line and a *Raphanobrassica* line (*R. sativus* x *B. napus*; 2n=56 AACCRR) (Heyn 1976). In *B. napus,* one dominant nuclear gene, *Rfo*, restores the fertility in hybrid plants by decreasing the accumulation of ORF138 protein in flower buds (Bellaoui et al. 1999). When the Ogura restorer lines of rapeseed were introduced, the large proportion of introgressed radish genome resulted in poor agronomic traits, such as reduced seed set (Pellan-Delourme and Renard 1988) and high glucosinolate content (Delourme et al. 1998). Intensive breeding and deletion of most of the radish introgression produced agronomically improved Ogura restorer lines of rapeseed (Delourme et al. 1991, 1995; Primard-Brissed et al. 2005). This modified Ogura system is also called the Ogu-INRA CMS/Rf system, indicating the improved CMS and Rf lines. In later studies, it was shown that the radish *Rfo* locus consists of three closely related PPR genes in tandem, *ppr*-A, *ppr*-B and *ppr*-C (Brown et al. 2003; Desloire et al. 2003), of which *ppr-B* is the fertility restorer (Brown et al. 2003; Desloire et al. 2003; Uyttewaal et al. 2008).

The Kosena CMS/Rf system was found in a population of the Japanese radish cultivar ‘Kosena’ (referred by Sakai et al. 1996) and was transferred first to *B. napus* by donor-recipient protoplast fusion (Sakai and Imamura 1992; Sakai et al. 1996). In subsequent research, it was shown that the Ogura and Kosena CMS/Rf systems were genetically the same. The Kosena

sequence of the CMS-associated gene, *orf125*, is homologous to that of the Ogura CMS-associated gene, *orf138*, except for two amino acid substitutions and a 39 bp deletion in the *orf138* coding area (Iwabuchi et al. 1999). Crossing studies, where both Ogura and Kosena CMS plants were crossed with Kosena maintainer and restorer plants, showed that the maintenance and restoration were the same for these two CMS systems. Koizuka et al. (2000) demonstrated that two dominant genes, *Rfk1, Rfk2*, restored fertility in radish but the *Rfk1* gene alone was sufficient for restoration in oilseed rape. The *Rfk1* gene was shown to regulate the level of ORF125 protein accumulation (Iwabuchi et al. 1999; Koizuka et al. 2000, 2003), and likewise the *Rfo* gene regulates the accumulation of ORF138 protein in the Ogura system. Later, Koizuka et al. (2003) cloned the *Rfk1* gene and found that it codes an ORF687 protein in the PPR family. At the same time, Brown et al. (2003) found that the Ogura *Rfo* gene encodes the same sequence of ORF687 protein and concluded that the fertility-restoring genes in both systems were identical.

The Ogura CMS was previously transferred from *B. napus* to *B. rapa* (Sovero 1987; Delourme 1994). As in other *Brassicas*, the Ogura CMS produced male-sterile *B. rapa* lines that were highly stable in various climatic conditions (Sovero 1987). The transfer of the fertility-restoring *Rfo* gene from *B. napus* (AACC) to *B. rapa* (AA) was, however, unsuccessful (F.Stoenescu, personal communication, Zeneca Seeds, Winnipeg, Canada, 1995) because the *Rfo* gene had been introgressed into the C genome of oilseed rape (Hu et al. 2008; Feng et al. 2009). The Ogura CMS was selected for this study because of its highly stable male sterility according to previous studies. This knowledge was supported by the research group's own breeding work when producing several Ogura female lines for the turnip rape hybrid program. The information about the similarities between Ogura and Kosena systems, and the supposed different location of the Ogura and Kosena fertility restorer genes in the *B. napus* genome, confirmed the selection of Kosena Rf for the study. It was suggested that the Kosena fertility restorer gene (*Rfk1*) was located on an extra-chromosomal piece of radish chromosome in the Japanese oilseed rape breeding lines that were included in the study (J. Imamura, personal communication, Plantech Research Institute, Yokohama, Japan, 2002). To establish a functional hybrid system for turnip rape, the combination of Ogura CMS and Kosena Rf was identified as the most promising option.

1.3 UTILIZATION OF INTERSPECIFIC CROSSES IN GENUS *BRASSICA*

Interspecific crosses and the addition of alien chromosomes are procedures that have been utilized by plant breeders in order to transfer agronomically

desirable genes between species and to broaden the genetic basis of cultivated crops especially in *Brassica.* The genomic relatedness of different *Brassica* species affects the success of crossing and the introduction of desirable genes or chromosome segments. The possibility of using interspecific and intergeneric hybridization in the *Brassica* genome is based partially on the homologous relationships between the different genomes in the *Brassicaceae*. According to The *Brassica rapa* Genome Sequencing Project Consortium (2011), the tribe *Brassiceae* shares a common hexaploid ancestor and there is a whole-genome triplication in the *Brassica* lineage relative to the *Arabidopsis* lineage. Radish (RR) and *B. rapa* (AA) genomes share large homologous regions, but the order or composition of these regions do not correspond (Shirasawa et al. 2011). Many agronomically important traits have been characterized among different *Brassica* species, and the possible interspecific and intergeneric hybridizations have been listed (see Seguin-Swartz et al. 1997 and Warwick et al. 2009). In many cases, natural crossing is successful without the need for additional techniques such as embryo rescue or protoplast fusion. The crosses between *B. rapa* (AA) and *B. napus* (AACC) are generally successful and hybrid fertility is known to be high (Metz et al. 1997; Leflon et al. 2006). According to Namai et al. (1980), crossing between *B. rapa* (AA) and *B. juncea* (AABB) is also successful, but that between *B. rapa* and *B.oleracea* (CC) is more difficult and that between *B. rapa* and *R. sativus* (RR) is only occasionally successful. The high level of genome similarity contributes significantly to the success of interspecific crosses (Leflon et al. 2006). However, difficulty in hybrid seed production is not restricted to taxonomic differences, because the individual plant, cultivar and environmental conditions also cause variation (Namai et al. 1980).

The transmission of traits of interest can be affected in crosses by the use of the recurrent parent as female or male (Metz et al. 1997; Leflon et al. 2006). In most cases, the transmission rate is higher through the female than through the male line (Quiros et al. 1987; Struss et al. 1991; Metz et al. 1997), but atypical results have also been found (Chèvre et al. 1991). Overall, the transmission rate of alien chromosomes has varied with an average of 21% (Quiros et al. 1987; Struss et al. 1991), and a range from 20% to lower than 50% (Leflon et al 2006; Budahn et al. 2008), although up to 100% transmission has been reported (Chèvre et al. 1991). These results have been explained by differences in fertility of the hybrids, in chromosome size, or in viability and competitiveness of gametes and embryos.

In practice, problems arise due to the instability of the alien introgressions. The valuable genes are usually transferred from donor parent to the recurrent parent using interspecific crossing followed by repeated backcrossing. In many cases, the first result is a monosomic or disomic addition plant that carries one or two copies of the alien chromosome originating from the donor parent (Quiros et al. 1987; Chèvre et al. 1991; Struss et al. 1991; Lee and Namai 1994; Kaneko et al. 2001, 2003; Peterka et al. 2004; Budahn et al. 2008). At this stage the alien chromosomes have

mostly been found to be unstable (Chèvre et al. 1991; Peterka et al. 2004; Wei et al. 2010). However, according to Budahn et al. (2008), stable introgressions could be produced when two homologous extra chromosomes behave normally in meiotic devisions. In some cases, stable disomic additions have been found from *B. napus* – *R. sativus* (Budahn et al. 2008), *B. napus* – *B. nigra* (Chèvre et al. 1991) and *B. napus* – *B. carinata* lines (Navabi et al. 2010). In *Brassica* species, the stability of the additional chromosomes could be regulated by epigenetic phenomena in the parental material (Ge and Li 2007; Ge et al. 2009). The similarity or difference in these components affects the chromosome structure and function during meiotic divisions in hybrids.

According to Chang and de Jong (2005), another way to produce stable introgressions is through meiotic recombination between the alien chromosome and one of its homeologues. The knowledge of the homeologous regions between the genomes increases the possibilities of transferring valuable traits through homeologous recombination (Lagercrantz and Lydiate 1996). Different mechanisms and genes control crossovers, and the increasing knowledge of these mechanisms assists plant breeders to transfer desirable genes between related species (Snowdon 2007; Nicolas et al. 2008; Wijnker and de Jong 2008). Nicolas et al. (2007) indicated that crossovers occur more frequently between the most related chromosomes, however, in the absence of these true homologous chromosome pair crossovers also occur between homeologous regions, e.g. the A and C chromosomes. In addition to the degree of relatedness, the frequency of crossovers is affected by the karyotype and genetic composition of the plant (Nicolas et al. 2007, 2008, 2009; Leflon et al. 2010). The homeologous recombination activity was much greater in *B. napus* haploids AC than in amphidiploids AACC (Nicolas et al. 2007, 2009). The hybrid karyotype composition influenced the crossover rate. The overall recombination activity was higher in the newly formed allotetraploid AACC hybrid than in the diploid AA hybrid, and was highest in the triploid AAC hybrid (Leflon et al. 2010). Mason et al. (2010) found that complex interactions between genomic structure and alleles derived from parental species control homologous and homeologous pairing in *Brassica* and it is more quantitatively than qualitatively inherited.

1.4 *IN SITU* HYBRIDIZATION TO STUDY CHROMOSOME COMBINATIONS

Fluorescence *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH) techniques have been widely used in plant chromosome and genome research (Schwarzacher and Heslop-Harrison 2000). In interspecific hybrids, the *in situ* hybridization methods have been very useful for detecting different genomes as well as locating introgressed genomes, alien chromosomes, or

chromosomal segments in another genomic background (Swarzacher et al. 1992; Raina and Rani 2001). In the FISH technique, any source of DNA and RNA can be used as a probe in hybridization, but cloned DNA sequences are most commonly used (Schwarzacher and Heslop-Harrison 2000). The use of bacterial artificial chromosome (BAC) clones as FISH probes has increased greatly in recent cytogenetic studies in genus *Brassica*. The *Brassica* genome-sequencing projects have provided useful sequence information (for review, see Heslop-Harrison and Swarzacher 2011) and the *Brassica* BAC clones have been used in numerous studies for chromosome identification as well as physically mapping the genes or specific sequences on chromosomes (Howell et al. 2005, 2008; Nicolas et al. 2007, 2008; Feng et al. 2009; Kim et al. 2009; Szadkowski et al. 2010, 2011; Xiong and Pires 2010; Xiong et al. 2011).

In the GISH technique, the whole genomic DNA from one parent is labelled and used as a probe in hybridization. GISH is most powerful when the evolutionary divergence between the studied genomes is adequate for separation. Transposable elements are abundant in *Brassica* genome and some of these are genome-specific (Alix et al. 2005, 2008; Lim et al. 2007). Some genome and chromosome specificity has also been shown in tandemly repeated DNA motifs in *Brassicas* (Harrison and Heslop-Harrison 1995). Nevertheless, the small chromosome size and limited evolutionary divergence of *Brassica* A, B and C genomes have limited the ability to distinguish them and their chromosomes with GISH (Snowdon et al. 1997). Between *Brassica* and *Raphanus*, however, the evolutionary distance is greater, and the radish R genome chromosomes have been successfully distinguished from the A and C genome chromosomes of *Brassicas* with GISH (Snowdon et al. 1997; Benabdelmouna et al. 2003; Chen and Wu 2008; Akaba et al. 2009). According to Sirasawa et al. (2011), the A genome of *B. rapa* and R genome of *R. sativus* share large homologous genomic segments, but the order and composition is different, making their separation robust.

2 AIMS OF THE STUDY

To utilize hybrid breeding in commercial scale, the plant species should have hybrid vigour or heterosis in valuable traits including increased seed yield, a functional hybrid seed production system and parent lines that combine well. The first aim of this study was to test the heterosis for seed yield in spring turnip rape with required quality parameters and adaptation for cultivation in short growing seasons. The second was to test the use of the Kosena fertility restoring gene *Rfk1* in spring turnip rape to generate a functional R-line for hybrid seed production together with Ogura CMS lines.

The specific aims were:

1) To study if spring turnip rape expresses heterosis in seed yield;
2) To study if the Kosena *Rfk1* gene, homologue to the *Rfo* gene, can be transferred from *B. napus* to *B. rapa* and generate the fertility-restoring R-line needed for turnip rape hybrid breeding;
3) To localize the *Rfk1* gene and the putative flanking region of radish chromatin in turnip rape genomic background, in order to evaluate the breeding methods needed to produce a functional hybrid system in turnip rape.

3 MATERIAL AND METHODS

3.1 PLANT MATERIAL

The plant material used for the yield potential study comprised a set of spring turnip rape parent lines, developed for the turnip rape hybrid program by the author and colleagues during the 1990s. The 22 parent lines selected for testing derived from 18 populations and crosses of Finnish, Canadian, Swedish and Russian origin. The breeding procedure is explained in publication I. All the parent lines were low in erucic acid (< 1.0%) and glucosinolate content (< 12 µmol g^{-1} of seed) and were well adapted for cultivation in northern areas. All the parent line combinations are described in paper I.

Plant material for the backcross program of the *Rfk1* gene from oilseed rape to turnip rape included the following. The donor parents were two spring oilseed rape breeding lines, RfA4 and RfA12, homozygous for the *Rfk1* gene, that were kindly provided by the Plantech Research Institute Japan. The three recurrent parents were spring turnip rape breeding lines, 4003, 4016 and 4021, which were selected from the hybrid program described above. All the turnip rape breeding lines were earlier backcrossed to Ogura CMS form.

The turnip rape fertility restoring line 4021-2 Rfk, developed in the backcross program (paper II), was used for the chromosome studies (paper III). The development of the restorer line 4021-2 Rfk is illustrated in Figure 1. The original breeding line 4021B with the same genetic background as 4021-2 Rfk, but without the radish restorer gene, was used as a negative control in this study.

3.2 METHODS

3.2.1 Field trials

Field trials for the turnip rape heterosis study were conducted during a three year period (2000-2002). The experiments were grown in a randomised complete block design with four replicates. Altogether five open-pollinated commercial cultivars, 16 synthetic cultivars and 25 composite hybrid combinations of parent lines were tested. The evaluated traits were height (cm), days to maturity, lodging at maturity stage (%), seed yield (ka ha^{-1}), oil (%) and protein content (%). Details of the experiment and evaluated traits are given in paper I.

3.2.2 Breeding methods

The traditional backcross method, followed by subsequent inbreeding and finally intercrossing of the homozygous plants, was used for transferring the Kosena *Rfk1* gene from oilseed rape to turnip rape. During the backcrossing, reciprocal crossings were used to study the transmission rate of the *Rfk1* gene trough the egg and pollen cells. Transmission of the *Rfk1* gene was followed by male fertility observations, which was verified by PCR analysis. A detailed description of backcross generations and male fertility evaluation is given in paper II.

3.2.3 PCR methods

The presence of the Ogura CMS and *Rfk1* genes was verified in the backcross program using PCR. The specific primer pairs were designed by Plantech Research Institute, Japan. The primer pairs for the *Rfk1* gene were based on the marker E90 (Imai et al. 2003). The detailed description of the PCR method is in paper II.

In selection of homo- and heterozygotic plants carrying the *Rfk1* gene, both testcross results and the TaqMan qPCR analysis were used. The Plantech Research Institute, Japan, kindly provided the TaqMan qPCR protocol for the study. The *Rfk1* gene, the target gene in this study, was quantified by using the primer pairs based on the DNA marker E90 (Imai et al. 2003). The BRTFL1-1 gene (TERMINAL FLOWER 1-like gene; AB017528.1), identified from *B. rapa* (Mimida et al. 1999), was used as the reference gene. The ratio of the normalized gene expression values between E90 and BRTFL1-1 were optimized so that in the homozygous situation it was double that in the heterozygous situation. The TaqMan qPCR analysis was done in two different generation of homozygote selection. In the first experiment, the analysed plants were inbred and the selection was done for the harvested seeds, but in the second experiment the selection was done prior to flowering and all homozygous plants were interpollinated and seeds bulked. The detailed descriptions of the TaqMan-based qPCR analysis and testcrosses are in paper II.

3.2.4 Chromosome studies

The physical location of the radish introgression carrying the *Rfk1* gene was investigated using BAC-FISH and GISH. For the *in situ* hybridization, the chromosome preparations were made from the root tips of turnip rape using standard methods (Schwarzacher and Heslop-Harrison 2000). The protocol is briefly described in paper III. The *in situ* hybridization was performed using

genomic radish DNA and radish BAC clone probes simultaneously. The DNA for the genomic probe was extracted from leaves of Japanese radish 'Daikon'. The BAC clone probe for the study, BAC64 (Desloire et al. 2003) from Genoplante-Valor, was kindly provided by INRA-Centre National de Ressources Genomiques Vegetales (CNRGV, Castanet-Tolosan, France). The studied fertility-restoring *Rfo* locus (homolog to *Rfk1*) is in *R. sativus* BAC64 (contig 127, accession number AJ550021). The genomic and BAC clone probes were labelled with biotin-11dUTP (Roche) and digoxigenin-11dUTP (Roche). As a control for the hybridization procedure, two ribosomal DNA probes, 5S and 45S, were used and labelled with Alexa-647-dUTP (Invitrogen). The detailed protocol for the probe labelling, *in situ* hybridization and signal detection is given in paper III.

3.2.5 Statistical methods

All the statistical methods used are described in the respective publications. The yield trials were analysed using the MIXED procedure of the SAS system (version 8.2, SAS Institute, Inc., Cary, NC) and the commercial heterosis was calculated according to Schuler et al. 1992 (paper I). The TaqMan qPCR values were calculated using the procedure presented by Muller et al. 2002 (paper II). In distinguishing the homo- and heterozygotic plants, two methods were used and the TaqMan qPCR and testcross results were analysed in quartet analysis. The statistical measures were calculated for the different classification rules set for the qPCR results. The statistical measures used were the agreement rate, false-positive rate, false-negative rate, Cohen's kappa-value and area under the ROC-curve (Fleiss et al. 2003) (paper II).

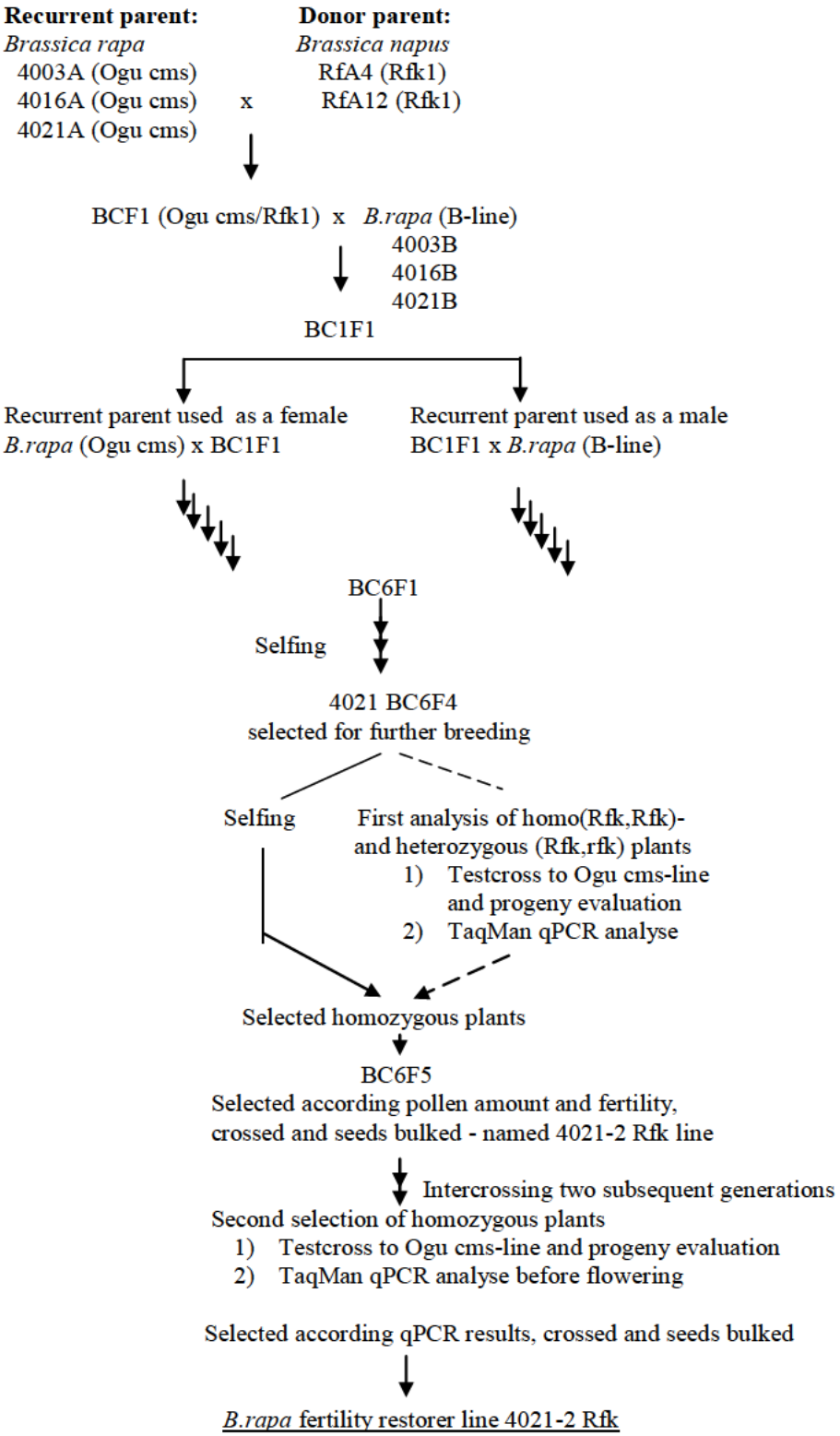

Figure 1 Illustration of the backcross breeding procedure transferring the *Rfk1* gene from *B. napus* to *B. rapa*

4 RESULTS

4.1 THE PERFORMANCE OF DIFFERENT CULTIVAR GROUPS AND EXPRESSION OF HETEROSIS IN SEED YIELD (I)

In comparisons of the open-pollinated, synthetic and composite hybrid cultivars, the differences within each cultivar group were significant in almost all studied parameters including seed yield, days to maturity, height and lodging. The seed yield, the main object of this study, in the open-pollinated group was 2093-2356 kg ha^{-1}, in the synthetic group 1957-2554 kg ha^{-1} and in the composite hybrid group 2065-2668 kg ha^{-1}. When comparing the mean values of different cultivar groups, most of the significant differences were found between the open-pollinated and the composite hybrid group. On average, the composite hybrids had higher seed yield (P=0.03) and therefore higher oil (P=0.02) and protein yield (P<0.001), but not higher oil or protein content than the open-pollinated group. The oil and protein contents were nearly equal between the groups. Composite hybrids matured on average 0.6 d later (P=0.02) and had better lodging resistance (P=0.05) than the open-pollinated group. Overall, all the tested cultivars matured in a four-day period and there was no significant correlation between maturation date and seed yield (r^2=0.0054). The synthetic group was the most lodging-resistant compared to both the open pollinated (P<0.001) and the composite hybrid groups (P=0.008). Lodging resistance and seed yield did not correlate (r^2=0.0551) and, some of the best yielding synthetics were also the most lodging-resistant ones.

The heterosis in seed yield was calculated using commercial heterosis, i.e., the heterosis over the well adapted commercial cultivars ‘Kulta’ and ‘Valo’ that were the most commonly used (over 80%) in 2000-2002. The commercial heterosis calculated for the synthetic group was 6% (P=0.10) and for the composite hybrid group 8% (P=0.03). The range in commercial heterosis for seed yield within synthetics was -10 to 18% and within the composite hybrids -5 to 23%.

4.2 BACKCROSSING THE *RFK1* GENE FROM *B. NAPUS* TO *B. RAPA* (II)

The Kosena fertility-restoring *Rfk1* gene was transferred from *B. napus* to *B. rapa* by traditional backcrossing followed by subsequent inbreeding and intercrossing of homozygous plants (Figure 1). Pollen production was normal in 83% of the F1 plants. Reciprocal crossing during the backcrossing from BC2F1 to BC6F1 showed that the transmission rate of the restorer gene was

slightly better through the female side from BC2 to BC3 and through the male side from BC4 to BC6. The average transmission rate of the restorer gene ranged between the backcross generations from 29% to 35%. When verifying the transmission of the *Rfk1* gene, the primer pairs for Ogura CMS and Kosena *Rfk1* gene were found to be specific, because no PCR product was detected in maintainer lines 4003B, 4016B and 4021B.

4.3 DETECTING HOMO- AND HETEROZYGOTE PLANTS BY TAQMAN QPCR (II)

After the restorer gene was backcrossed into turnip rape, all the breeding lines, 4003, 4016 and 4021, were inbred until BC6F3 or BC6F4 generation to get the restorer gene *Rfk1* into a homozygous condition. The breeding line 4021 showed the best seed production and vigour during the inbreeding generations so it was selected for further studies. The other two lines, 4003 and 4016, suffered from inbreeding depression and the viability and seed production of the offspring were too low to continue.

In the two experiments of distinguishing homozygous restorer plants from heterozygous ones, the highest agreement rate for the assessed cases was 98.4% (first qPCR) and 100% (second qPCR) indicating almost complete agreement between the testcross and the TaqMan qPCR method. The false positive rate was 0% in both cases and the false negative rate was 6.7% (first qPCR) and 0% (second qPCR). The rate of lost plants that were recorded as unknown was 8.5% (first qPCR) and 9.1% (second qPCR). The highest values of the receiver operating curve (ROC), an index of the probability of correct pair wise ranking was 0.97 (first qPCR) and 1.00 (second qPCR).

4.4 FUNCTION AND INSTABILITY OF THE *RFK1* GENE IN TURNIP RAPE GENOME (II)

During the detection of homozygous (Rfk,Rfk) and heterozygous (Rfk,rfk) plants, the testcross result itself indicated the unstable nature of the restorer gene in the genomic background of turnip rape. In the first experiment the male-fertility results of the testcross progeny varied in the heterozygous condition mostly from 20% to 40% and in the homozygous condition from 80% to 90% instead of the normal single dominant gene situation where the expected values would be 50% and 100% respectively. After the homozygous plants were selected, they were intercrossed for two subsequent generations and the fertility of the resulting turnip rape restorer line 4021-2 Rfk dropped from 100% to 83%. The second selection of homozygous plants was realized and the male-fertility results of the testcross progeny varied in the

heterozygous condition from 16.9% to 39.4% and in the homozygous condition from 92.1% to 100%. Although the *Rfk1* gene appeared to be unstable in the turnip rape genome, some testcross progeny with 100% male fertility was also found, demonstrating the ability of the *Rfk1* gene to restore fertility in turnip rape with Ogura CMS.

4.5 PHYSICAL LOCATION OF THE RADISH CHROMOSOME REGION CARRYING THE *RFK1* RESTORER GENE IN TURNIP RAPE (III)

4.5.1 *In situ* hybridization

GISH showed that the 4021-2 Rfk plants were either monosomic (2*n*=2*x*=20+1*R*) or disomic (2*n*=2*x*=20+2*R*) addition lines with one or two copies of an alien chromosome originating from *Raphanus*. The control line without the restorer gene did not show any hybridization signal. The BAC-FISH showed clear double dots of a hybridization signal, one on each sister chromatid of the radish chromosome identified by GISH. These two signals of the BAC64 probe were located in the subterminal parts of the radish chromosome arm. The control line 4021B showed no strong signals.

4.5.2 Homology of the BAC64 and the whole genome of *B. rapa* subsp. *pekinensis*

In addition to the clear hybridization signals of the BAC64 probe on radish chromosomes, two pairs of weaker signals were sometimes observed on two chromosomes of the turnip rape A genome. BLAST analysis compared the full-length sequence of the *R. sativus* BAC64 clone (AJ550021.2) with the whole genome of *B. rapa* subsp. *pekinensis* (The *B.rapa* Genome Sequencing Project Consortium 2011) and identified high homology with *B. rapa* subsp. *pekinensis* BAC clone KBrB025K04 (AC189288.2). This BAC KBrB025K04 is situated on the largest chromosome of the A genome, on the linkage group A09 (6.10.2011). Homology of 90% was found between the *R. sativus ppr-B* gene (*Rfo*/*Rfk1* gene) on BAC64 and the *B. rapa* fertility restorer gene (*Rf*; pentatricopeptide repeat-containing protein, fertility restorer B; KBrB025K04CG0180) on BAC KBrB025K04.The radish *Rfo* locus consists of three closely related PPR genes in tandem, *ppr-A*, *ppr-B* and *ppr-C,* and when the homology between *R. sativus* BAC64 and *B. rapa* linkage group A09 was tested in dot-plot matrix, the homology of all three PPR genes were shown. Moreover the dot-plot matrix demonstrated that the region covering all three PPR genes has two copies in *B. rapa* background.

5 DISCUSSION

Early maturing turnip rape is still the most important oilseed crop in northern agricultural areas. In Finland its cultivation exceeds 80% of the total rapeseed production area (http://www.maataloustilastot.fi/en/crop-production-statistics). Oilseed rape cultivation has increased during the last ten years (2001-2011) from 1.5% to 16% of the country's rapeseed production area due to the success of breeding for earliness, but its cultivation is still limited to the southern parts of Finland. In the national grain production strategy for 2006-2015, the targeted growing area for oilseed crops exceeds 100 000 ha. In order to reach that goal, the cultivation of rapeseed should be maintained on an extensive area and further gains in earliness are needed. The main challenge in turnip rape cultivation is the low seed yield. The average yield over last ten years (2001-2011) has been only 1330 kg ha^{-1}, limiting the economy of its production.

Hybrid breeding, with its effect of increased seed yield, is utilized commercially in many plant species such as oilseed rape. Breeding companies working on oilseed rape releases new hybrid cultivars every year, whereas turnip rape breeders still lack a functional hybrid seed production system. As a first step towards using heterosis in turnip rape, synthetics and composite hybrids were studied, and heterosis was found in seed yield, in oil and protein yield, but not in oil or protein content. The better lodging resistance of synthetics and composite hybrids was evident.

The increased yield potential of composite hybrids compared to open-pollinated cultivars is encouraging for hybrid breeding in this crop. The synthetics as a group also yielded more than the open-pollinated cultivars, but the difference was not statistically significant. Overall the group differences between synthetics, composite hybrids and open pollinated were rather low. Higher differences were measured within the groups in different parent combinations, in agreement with other experiments (Falk et al. 1994; Ofori and Becker 2008).

Overall the composite hybrids yielded 8% and the synthetics 6% more than the most widely cultivated cultivars. The tested parent line material showed clear differences in combining abilities. The range in commercial heterosis for seed yield varied both within synthetics and within composite hybrids. The best commercial heterosis for seed yield was 18% in synthetics and 23% in composite hybrids. Some heterosis values were negative, confirming that hybrid breeding in itself does not assure positive results. When making the homozygous parent lines in turnip rape, the inbreeding depression could decrease the vigour and seed production of the breeding line. In case the parent lines do not combine well, the more heterotic open-pollinated cultivars exceed them in seed production and negative values are shown in commercial heterosis. In this study the heterosis results for seed yield were consistent with previous studies in turnip rape; 10-14% midparent

heterosis of Syn1 (Falk and Woods 2003), 13% midparent heterosis of F1 hybrids (Falk et al. 1994), 23% midparent heterosis of Syn1 and 25% midparent heterosis of F1 hybrids (Falk et al. 1998) and 24% commercial heterosis of F1 hybrids (Schuler et al. 1992).

The commercial heterosis is less frequently used than midparent or highparent heterosis because it lacks a genetic basis, but it provides a useful benchmark. In this case the commercial heterosis was used in order to study larger number of parent lines with different breeding techniques. According to common knowledge heterosis should be tested in field conditions, because of the great influence of different environments. In testing the combining abilities of possible breeding lines, one could reduce the work by using a few parents with good general combining ability. Here, primarily two different testing parents were used in order to test as many parent lines as possible and to find the best combinations.

The parent lines tested all had good seed quality characters including low erucic acid (< 1%) and glucosinolate (< 12 μmol g^{-1} of seed) content and were well adapted to the short Finnish growing season. In previous studies, the highest values of heterosis were often measured when strains with poor seed quality characters, like high erucic acid and high glucosinolate content, were tested (Hutcheson et al. 1981; Schuler et al. 1992; Falk et al. 1994, 1998; Falk and Woods 2003). According to the classical theory, the level of heterosis is increased when the genetic distance of the parent lines increases (Chen 2010). To find more distant parent lines for testing, one could compromise in some agronomically important traits. From the breeder's point of view, however, it is very important that heterosis is achieved in the breeding lines selected according to the prevailing criteria, as was the case in this study.

Turnip rape, as a cross-pollinating crop, presents challenges for producing genetically different but highly inbred parent lines for hybrid breeding, in comparison with self-compatible oilseed rape. According to Schuler et al. (1992), the high degree of heterozygosity and strong inbreeding depression complicates the combining ability tests of turnip rape parent lines. In this study, the high degree of variation in almost all the studied traits, especially in yield potential between different parent combinations, confirms the importance of testing the parent combinations also in turnip rape.

Heterosis effects could be seen in increased growth and vigour followed by increased plant height and longer growing period (Brandle and McVetty 1990; Schuler et al. 1992). All the tested combinations were well adapted to the short growing period of northern latitudes and matured in a four-day period. The 0.6 days longer growing period of composite hybrids did not correlate with higher seed yield and supported the hypothesis that seed yield could be increased without lengthening the growing period. The more vigorous growth of synthetics and composite hybrids did not diverge in height from the open-pollinated cultivars but they showed significantly better lodging resistance. In previous studies with turnip rape (Hutcheson et al. 1981) and oilseed rape (Sernyk and Stefansson 1983; Busch 1995), the lodging

resistance of hybrids has been reported to be similar to or better than the parents.

The work to produce a functional, fertility-restoring (Rf) line for turnip rape in the Ogura/Kosena CMS/Rf hybrid seed production system was started by successfully backcrossing the Kos *Rfk1* gene from spring oilseed rape into spring turnip rape. The first cross was made using the recurrent parent, the turnip rape breeding line with Ogu CMS, as female and the donor parent, the oilseed rape breeding line with the *Rfk1* gene, as male parent. In this way, it was possible to observe the phenotypes of offspring plants carrying the restorer gene, although the PCR method verified the presence of both the Ogu CMS and the *Rfk1* gene in every backcross generation. The initial hybrid between *B. rapa* (AA) and *B. napus* (AACC) was easily made by cross-pollination and the male fertility of the F1 hybrid was good (83%), which agrees with the previous findings of crossing these species (Metz et al. 1997; Leflon et al. 2006).

The transmission rate of the *Rfk1* gene was studied using reciprocal crossing through BC2 to BC6 generation. It has been shown earlier that in interspecific crossing between *B. napus* and *B. rapa* the transmission of traits in successive generations of backcrossing can be affected whether the recurrent parent has been used as a female or as a male parent (Metz et al. 1997; Leflon et al. 2006). Here the *Rfk1* gene was transferred through both the egg and pollen cells, with very slight differences between directions of crossing, 35% through the pollen and 33% through the egg cells. Usually the transmission is higher through the female than through the male side (Quiros et al. 1987; Struss et al. 1991; Metz et al. 1997), but variability also exist (Chèvre et al. 1991). The gametic competition between male gametes is higher than between ovules leading to higher transmission rates through ovules (Leflon et al. 2006). In interspecific crosses the additional chromosome could lower the viability and competitiveness of male gametes (Chèvre et al. 1991; Leflon et al. 2006). However, it has been shown that the transmission rate and male fertility varies according the parental genotype and the different additional chromosome (Chevre et al. 1991; Leflon et al. 2006). The fitness of the parents, that is mostly genotypically determined, and the genetic effect of the specific chromosome affect to the transmission rate. In this study the fitness of the additional radish chromosome seems to be good in the background of the *B. rapa* line. The viability and competitiveness of male gametes showed no reduction in relation to normal euploid A genome gametes.

In the Kosena hybrid system in oilseed rape, one dominant *Rfk1* gene restores the male fertility in crosses with male-sterile Kosena and Ogura CMS plants (Koizuka et al. 2000). In the case of a single dominant gene the expected ratio in backcrossing would be nearer 50% than the 30% found here. The Kosena restorer breeding lines (RfA4, RfA12) of oilseed rape were selected for this study on the understanding that the fertility restorer gene *Rfk1*, originating from radish and homologue of the Ogura restorer gene (*Rfo*; Brown et al. 2003), was not integrated into the C genome (J. Imamura,

personal communication, Plantech Research Institute, Yokohama, Japan, 2002). Nevertheless, it was apparent that one contributing factor to the lack of success in transferring the *Rfo* gene from oilseed rape to turnip rape was the introgression of the *Rfo* gene into the C genome (Hu et al. 2008; Feng et al. 2009). The fairly stable 30:70 segregation ratio, in the present study, indicated a possible aneuploid condition of an extra chromosomal piece of radish chromosome, which was later proved to be the case.

During the breeding work, it was observed that some of the turnip rape breeding lines suffered from the inbreeding depression that is common in cross-pollinating crops. In order to avoid it, the TaqMan-based qPCR was used to discriminate homozygote from heterozygote in segregating progeny. The selection was done before the flowering stage, which enabled interpollination instead of inbreeding of the selected homozygous plants. The TaqMan qPCR method has been effectively used also in the early screening of transgenic homozygotes in a segregating population of cotton (Yi et al. 2008). When introducing new traits between plant species, the allelic selection analysis of TaqMan qPCR is useful. The TaqMan qPCR method is based on a specific fluorogenic probe and enables the screening of copy number of target genes. In our study, the qPCR results were shown to be sensitive for the selected control DNA. Here, two different control DNAs were used in the experiments. Although the control DNAs were based on progeny of sister lines, they reacted slightly differently in qPCR. This caused variation when calculating the mean NE ratios of sample and control, but the difference was constant through each experiment. The problem was solved by setting the limits for the classification rules in each experiment according to the frequencies of homo- and heterozygous plants at different mean NE ratios, and the selection was based on that. The qPCR results can vary between samples for several reasons, but if the variation is consistent the method can be reliable (Bustin et al. 2009). When setting up the analysis, comparison of the qPCR results with testcross results is recommended. Here the selection based on qPCR results agreed well with the genotype results obtained by testcross progenies.

The ability of the Kosena Rf gene or genes to restore the male fertility in Ogura CMS has been tested in previous studies with radish and oilseed rape (Iwabuchi et al. 1999; Koizuka et al. 2000). The Kosena and Ogura CMS/Rf systems are genetically the same and the maintenance and restoration are also the same (Iwabuchi et al. 1999; Koizuka et al. 2000). During the course of this breeding program, it was observed that the Kosena *Rfk1* gene restored the fertility in turnip rape with Ogura CMS, which agreed with the results with radish and oilseed rape. Although the genetics of fertility restoration was not studied here, several testcrosses were done between turnip rape Kosena restorer plants and the male sterile Ogura CMS plants. In the final selection of homozygous restorer plants, the testcrosses gave male fertility results from 90% to 100%. It was concluded that the imperfect fertility was due to instability of the *Rfk1* gene in turnip rape background.

The unstable nature of the Kosena restorer gene in the turnip rape genome was shown when after subsequent selection and interpollination of homozygous restorer plants, the progeny also had some heterozygous and male-sterile plants. The instability of the genes transferred through interspecific crosses in the genus *Brassica* has been reported in several studies (Chevre et al. 1991; Peterka et al. 2004; Wei et al. 2010). *In situ* hybridization was used for localizing the restorer gene in turnip rape A genome and to understand the nature of the instability for further breeding methods towards the stable restorer line for turnip rape.

The *in situ* hybridization using GISH and BAC-FISH defined the physical position of a chromosome region containing the fertility restorer gene *Rfk1* located on an additional radish chromosome in the A genome of spring turnip rape. The genomic radish probe hybridized to the additional chromosomes, showing both monosomic and disomic additions in the fertility-restoring turnip rape line 4021-2 Rfk. The restorer line was previously selected for homozygosity, and since both monosomic and disomic additions appeared, the unstable nature of this additional radish chromosome was demonstrated. In a further two generations of selecting and intercrossing 100% homozygous plants (analyzed by TaqMan qPCR), the progeny segregated to 90% homozygous and 10% hemizygous plants (data not shown). The instability of the restorer is minor, but in commercial hybrid seed as well as in a new R-line production, stability of the restorer gene is essential.

In previous studies, the disomic additions of radish chromosomes in rape-radish lines were expected to be stable (Budahn et al. 2008) and in studies with *B. napus – B. nigra,* stable disomic additions have been described (Chèvre et al.1991). Here, however, the disomic addition of radish chromosome proved unstable in turnip rape. From the practical point of view, genetic instability is the major problem in utilizing useful traits through alien additions. Unstable chromosome additions have been described in several studies when intercrossing different *Brassica* species (Chèvre et al. 1991; Peterka et al. 2004; Wei et al. 2010).

The stability of the *Rfo* gene and the *Rfk1* gene in oilseed rape has been achieved through the integration of the restorer gene into a C genome chromosome (Sakai et al. 1996; Primard-Brissed et al. 2005; Hu et al. 2008; Feng et al. 2009). To develop a stable Kosena restorer line for turnip rape, the integration of the *Rfk1* gene into an A genome chromosome through homeologous recombination may be required.

The BAC clone probe, BAC64 carrying the *Rfo* locus, homologue to *Rfk1*, identified the locus in both *Brassica* and *Raphanus*. The 90% homology between the studied *R. sativus ppr-B* gene, that has the fertility-restoring ability in the *Rfo* locus, and the *B. rapa* ssp. *pekinensis* KBrB025K4CG0180 gene showed that these genomic regions are homologous between A and R genomes. According to Mora et al. (2010), these two restorer genes are homologous, except the *B. rapa* restorer gene *B* (KBrB025K04CG0180) is unable to restore the fertility. The *Rfo* locus as a whole consists of three closely related PPR genes (*ppr-A*, *ppr-B* and *ppr-C*) in tandem and the

homologies between A and R genomes of all these PPR genes were shown in the dot-plot matrix analysis. It is well known that the sequence similarity increases the possibility of homeologous recombination. The BAC64 clone was located in the subterminal region of both the additional radish chromosome and the turnip rape chromosome (A09). Intergenomic introgressions often occur at distal parts of the chromosomes (Kim et al. 2002; Wang et al. 2007) indicating that these chromosome areas could have higher recombination activity. In the present study, the homology of the fertility-restoring region found between A and R genome and its location in the distal part of the chromosomes facilitate the opportunity to transfer the Kosena restorer gene *Rfk1* from the additional radish chromosome to a turnip rape chromosome by homeologous recombination.

The high level of genetic relatedness promotes the potential for homeologous recombination between *Brassica* species (Leflon et al. 2006). According to a recent study, *R. sativus* and *B. rapa* share large homologous genomic regions but the order or composition of these segments do not correspond (Shirasawa et al. 2011). The added radish R chromosomes have mostly stayed unaltered in the background of *Brassica* A and C genome (Peterka et al. 2004). The genetic information that regulates homologous pairing during meiosis should be interrupted to favour recombination between non homologous A and R genomes. The knowledge of the mechanisms and genes that control crossovers is increasing and would provide useful tools for plant breeders to promote homeologous recombination in exploiting targeted traits in between different species (Snowdon 2007; Nicolas et al. 2008; Wijnker and de Jong 2008).

In previous studies, when the Ogura (*Rfo*) or Kosena (*Rfk1*) restorer genes were transferred from radish to oilseed rape, the homeologous recombination between R and C genome were induced by using polyploid crosses (Heyn 1978), gamma irradiation of donor pollen (Primard-Brissed et al. 2005) or asymmetric protoplast fusion (Sakai et al. 1996). According to Nicolas et al. (2009) and Leflon et al. (2010), the homeologous recombination is dependent not only on the degree of genetic relatedness but also on the plant karyotype and genetic composition. They found that change in plant ploidy level could increase homeologous pairing in *Brassica*. In the present case, it would be worth studying if a crossing program with a triploid hybrid of oilseed rape breeding line RfA4 (having *Rfk1* gene) and turnip rape breeding line 4021-2 Rfk could increase the overall homeologous recombination between A, C and R genome chromosomes. The irradiation technology used successfully in developing the R2000 Ogu-INRA restorer line for oilseed rape (Primard-Brisset et al. 2005), could also promote the recombination between the R and A genomes.

6 CONCLUSIONS

Spring turnip rape (*Brassica rapa* ssp.*oleifera*) expressed heterosis for increased seed yield. Composite hybrids showed significantly higher yield potential than open-pollinated cultivars, supporting the objective of hybrid breeding to increase yield in this crop. Testing the combining abilities of the parent lines is important in turnip rape. A high degree of variation in commercial heterosis for seed yield was observed between the tested combinations within the groups in both synthetics and composite hybrids. The highest yield increase achieved in this study was 18% in synthetics and 23% in composite hybrids.

All the parent line material tested in this study had good seed quality characters including low erucic acid (<1%) and glucosinolate (<12µmol g^{-1} of seed) content, so it is possible to achieve heterosis with breeding material that meets the quality requirements. The tested material was also well adapted to the short growing season of northern climates. With hybrid breeding, it was possible to increase turnip rape seed yields up to 20% without lengthening the growing period. The positive heterosis for agronomically important lodging resistance was clear in both synthetics and composite hybrids compared to open-pollinated cultivars.

The heterosis for seed yield was evident although 100% F1 hybrids were not tested. This study was part of the turnip rape breeding program and heterosis effects were tested on synthetics and composite hybrids, where the proportion of F1 hybrid plants was about 50% and 75% respectively. The yield advantage of 100% F1 hybrids could be higher than that shown here, although the yield increase of 20% is remarkable and supports the use of F1 hybrids in spring turnip rape.

To produce a functional R-line for turnip rape hybrid system, the Kosena fertility- restoring *Rfk1* gene, originating from radish and homologue to the Ogura fertility-restoring gene *Rfo*, was transferred successfully from oilseed rape (*B. napus*) to turnip rape. Transferring of the gene was realized using interspecific crosses followed by traditional backcrossing. The *Rfk1* gene was transferred both through the egg and pollen cells, with a transmission rate around 30%.

The TaqMan-based real-time qPCR analysis was proved to be useful in selecting homozygous plants carrying the *Rfk1* gene originating from radish (*Raphanus sativus*). The TaqMan qPCR method was validated by comparing the results with the zygosity results according to the testcross progeny. Selecting the homozygous plants before flowering enabled the use of interpollination instead of inbreeding. Cross-pollinating crops can suffer severe inbreeding depression, making the breeding work more difficult. The TaqMan qPCR method allows effective and early detection of homozygous plants from the segregating population.

During the course of the breeding program, it was observed that the *Rfk1* gene was able to restore the fertility in turnip rape with Ogura CMS. Therefore, the *Rfk1* gene could be used for effective fertility restoration in turnip rape hybrid production. However, the trait was unstable in the turnip rape genome, even when the disomic addition of radish chromosome was selected.

The Kosena fertility restorer gene *Rfk1* was localized on the additional radish chromosome in the A genome of turnip rape. Generating a stable restorer line for turnip rape would require integration of the restorer gene into an A genome chromosome. The BAC64 clone, carrying the *Rfo* locus, homologue to the *Rfk1*, identified the locus both in the additional radish chromosome and in a turnip rape chromosome (A09). The high homology of this locus between radish and turnip rape and the location in subterminal part of the chromosome in both genomes would facilitate the transfer of the fertility-restoring trait from radish to turnip rape. It was concluded that additional breeding techniques may be required, such as changing the ploidy level or using irradiation to increase the recombination activity between non homologous R and A genomes.

The results of this thesis support the use of hybrid breeding in producing higher-yielding turnip rape cultivars. The breeding work towards a stable, fertility-restoring male line for the Ogu-INRA CMS/Rf hybrid system should be continued. To have a functional Ogu-INRA CMS/Rf hybrid system for turnip rape, it seems ideal if the homologous chromosomal area in the A genome could be substituted with the radish chromosome area having the restorer gene.

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